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Matilde Tschon
Serena Incerti-Parenti
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Photobiomodulation with low-level diode laser promotes osteoblast migration in an *in vitro* micro wound model

Matilde Tschon,[a] Serena Incerti-Parenti,[b,*] Simona Cepollaro,[a,c] Luigi Checchi,[d] and Milena Fini[a]
[a]Rizzoli Orthopaedic Institute, Laboratory of Preclinical and Surgical Studies, via di Barbiano 1/10, Bologna 40136, Italy
[b]University of Bologna, Unit of Orthodontics, Department of Biomedical and Neuromotor Sciences, via San Vitale 59, Bologna 40125, Italy
[c]University of Bologna, Department of Medical and Surgical Sciences, via Massarenti 9, Bologna 40138, Italy
[d]University of Bologna, Unit of Periodontology, Department of Biomedical and Neuromotor Sciences, via San Vitale 59, Bologna 40125, Italy

Abstract. Laser photobiomodulation can improve bone healing, but well-defined treatment parameters are lacking. Saos-2 human osteoblast-like cells were subjected to an *in vitro* scratch-wound healing assay and irradiated by a 915-nm gallium-aluminum-arsenide diode laser for 0, 48, 96, and 144 s using doses of, respectively, 0, 5, 10, and 15 J/cm^2. Wound area was measured after 4, 24, 48, and 72 h. Cell viability, DNA content, gene expression, and release of bone-related proteins were evaluated after 24, 48, and 72 h. Laser significantly improved wound healing compared with nonirradiated controls. Cells treated with laser doses of 5 and 10 J/cm^2 reached wound closure after 72 h, followed by 15 J/cm^2 after 96 h. With the cell proliferation inhibitor Mitomycin C, the doses of 10 and 15 J/cm^2 maintained an improved wound healing compared with controls. Laser increased collagen type 1 gene expression with higher doses inducing a longer-lasting effect, whereas transforming growth factor-beta 1 showed comparable or decreased levels in irradiated versus nonirradiated groups, with no effect on protein release. This study demonstrated that laser photobiomodulation at 915 nm promoted wound healing mainly through stimulation of cell migration and collagen deposition by osteoblasts. [DOI: 10.1117/1.JBO.20.7.078002]

Keywords: photobiomodulation; low-level laser therapy; diode laser; osteoblast-like cells; in vitro scratch assay; wound healing.
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1 Introduction

Photobiomodulation (PBM) has recently been defined as a form of light therapy that utilizes nonionizing forms of light sources, including lasers, light-emitting diodes (LED), and broadband light, in the visible and infrared spectrum. It is a nonthermal process involving endogenous chromophores eliciting photophysical (i.e., linear and nonlinear) and photochemical events at various biological scales.[1] PBM with low-level laser, which falls within this comprehensive definition, promotes various beneficial effects, such as reduction of inflammation, alleviation of pain, and acceleration of tissue repair.[2] The mechanisms underlying these therapeutic outcomes are not fully understood, but it is thought that PBM modulates cellular metabolic processes via a nonthermal action, leading to an enhanced tissue regenerative potential due to stimulation by light alone.[2] Wound healing is one of the areas of main interest for PBM because laser promotes healing and reduces pain at the same time.[3] PBM in musculoskeletal tissues has therapeutic benefits for the treatment of pain, osteoarthritis, and tendinitis.[4,5] It is also able to accelerate the process of bone formation as well as the healing of bone defects, fractures, and delayed consolidation; PBM can counter the process of bone resorption in osteoporosis acting as a rebalancing factor for proper bone remodeling.[6–9] Despite considerable improvements in the development of surgical treatments, bone substitutes or adjuvant therapies, such as ultrasonic treatment and pulsed electromagnetic field,[10–12] providing optimal tissue healing and improving the quality of life of patients, remain a challenge for orthopedic, maxillofacial and oral surgery. In this context, PBM is a highly promising strategy because it is accessible, easy to administrate, safe, painless, does not require the concomitant use of drugs, and may be also applied in the presence of metal devices.[8] Many clinical and experimental studies have investigated the influence of PBM on bone fracture healing,[8,13–15] tendinopathy,[16,17] osteoarthritis,[18] alveolar bone healing after tooth extraction,[19] and bone regeneration in the midpalatal suture after maxillary expansion.[20] Moreover, *in vivo* experimental studies have assessed the effects of laser irradiation on oral wound healing.[21–26] However, the wide range of laser sources, optical parameters and application times used in the many different studies make comparisons between studies very difficult and definite treatment protocols for the clinical practice almost impossible to be extrapolated. *In vitro* assays are, thus, required in order to overcome these problems because PBM parameters may be evaluated more rigorously at a cellular level as a first step toward standardization of treatment protocols for following preclinical and clinical applications.

*Address all correspondence to: Serena Incerti-Parenti, E-mail: serena.incerti2@unibo.it

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So far, the effect of PBM in the process of *in vitro* wound healing has been evaluated on adenocarcinoma human alveolar epithelial cells, rat kangaroo renal epithelial cells,[27] oral keratinocytes,[28] human gingival fibroblasts,[29] human gingival epithelial cells,[30] diabetic wounded fibroblasts,[31] human epidermal stem cells,[32] and tenocytes.[33] Only one study has investigated the effect of laser and LED sources on human cells derived from an osteosarcoma line using U2OS cells, finding that both sources enhance wound closure and concluding that biochemical and functional investigations on the mechanism of action and downstream pathways are needed.[27] However, U2OS cells are negative for most osteoblast markers and are considered closer to fibroblasts than osteoblasts.[34,35]

The aims of the current study were (1) using an *in vitro* scratch-wound healing assay to analyze the influence of irradiation by a gallium-aluminum-arsenide (GaAlAs) diode laser with a wavelength of 915 nm on the migration and proliferation of Saos-2 osteoblast-like cells, which resemble human mature osteoblast phenotype, play a key role in bone remodeling, differentiate, and form calcified matrix, (2) to compare the effects of different doses of 5, 10, and 15 J/cm^2 of laser irradiation, and (3) to evaluate modulation by laser irradiation of the differential gene expression and release of bone metabolism related proteins. The relative contribution of cell proliferation and migration to the scratch-wound closure was also investigated using the cell proliferation inhibitor Mitomycin C (MMC).

2 Materials and Methods

2.1 *Cell Culture*

Saos-2 human osteoblast-like cells (ATCC® HTB-85™) were cultured in Dulbecco modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, Missouri) enriched with 10% fetal calf serum (Lonza Walkersville Inc., Walkersville), 100 IU/ml penicillin, and 100 μg/ml streptomycin solution (Gibco Invitrogen SRL, San Giuliano Milanese, Milan). When confluent, cells were detached with 0.05% (w/v) trypsin and 0.02% (w/v) ethylenediamine tetra-acetic acid (EDTA), counted and seeded into black 24-multiwell tissue culture plates with clear bottoms (STEPBIO S.r.l., Bologna, Italy) at a density of 2.6×10^4 cell/cm^2. Plates were returned to the controlled humidified incubator (37°C in temperature, 95% air/5% CO_2).

2.2 *In Vitro Micro Wound Model*

After confluence had been reached, Saos-2 cultures were wounded with a sterile 200-μm Eppendorf tip to create a cell free zone in the monolayer (at baseline, T0, wound area measured 7.2 ± 0.4 mm^2). Cells were extensively washed with sterile phosphate buffered solution (PBS, Gibco Invitrogen SRL, San Giuliano Milanese, Milan), then laser irradiated. To discriminate the contribution of cell proliferation and migration to the process of wound closure, half of the wells were treated with MMC (Sigma-Aldrich) at a concentration of 50 ng/ml. Cell cultures were incubated and observed with an inverted microscope (Nikon Eclipse Ti-U, Nikon Italia, Italy) equipped with a digital camera (Sight DS-Fi2, Nikon Italia, Italy) after 4, 24, 48, 72, and 96 h from laser irradiation. Each well was photographed at 4× magnification to cover the wounded area. The image acquisition software (NiS Elements Advanced Research, Nikon Italia, Italy) was used to measure the area of the cell free zone of the artificially created wounds.

2.3 *Laser Irradiation*

Cells were exposed to irradiation with a GaAlAs diode laser (Pocket Laser, Orotig s.r.l., Verona, Italy), which has a wavelength of 915 ± 10 nm and a maximum power output of 6 W $\pm$ 20%. A 100 Hz pulse irradiation mode, a duty cycle of 50%, and a set power of 1 W (corresponding to an output power of 0.575 W, as measured at hand piece aperture) were used for 48, 96, and 144 s. The administered doses were, respectively, of 5, 10, and 15 J/cm^2. The laser beam was delivered perpendicularly to each well by an optical fiber 0.6 mm in diameter that was defocused at the tip by a concave lens to cover the growth area of each well (1.91 cm^2) at a distance of 19 mm. To avoid overlapping or scattered irradiation, black multiwell plates were used for all the assays. During the period of laser irradiation, the cover plate was removed and DMEM was replaced with PBS to avoid serum interference during irradiation.[2] Control cells were not irradiated. Both control and laser-treated cells were cultivated under the same experimental conditions.

2.4 *Viability Test and DNA Quantification*

Alamar Blue assay (AbD Serotec, Oxford, United Kingdom) was used to evaluate cell viability after 24, 48, and 72 h from laser irradiation. Alamar was added to each culture well (1:10 v/v) for 4 h at 37°C. The colorimetric reaction was measured spectrophotometrically on supernatants at 570 and 625 nm wavelengths with a microplate absorbance reader (iMark, Biorad-Laboratoires Inc., Hercules, USA).

DNA quantification (Quant-iT™ PicoGreen® dsDNA) was performed following manufacturer's instructions. Briefly, cells were repeatedly washed with PBS, frozen at −80°C, and thawed at room temperature three times. Cell lysis was obtained by adding 100 μl of Tris-EDTA buffer with sodium dodecyl sulphate 0.01% solution. A working solution of the PicoGreen® reagent was added and incubated with cell lysates in the dark for 3 min at room temperature. The fluorescence was read at 490ex-520em wavelengths, the readings were expressed as relative fluorescence units, and the DNA amount of each sample was calculated above a standard curve.

2.5 *Quantification of mRNA Expression Levels by Quantitative Polymerase Chain Reaction*

After 24, 48, and 72 h from laser irradiation, Saos-2 cells grown in the presence of DMEM (10% fetal bovine serum, 1% penicillin-streptomycin and plasmocin) were homogenized and total RNA extraction was performed by the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Total RNA was eluted with RNase-free water, quantified by NanoDrop 2000 (Thermo Scientific, Waltham, Massachusetts), and kept at −80°C until reverse transcription.

Each RNA sample (2500 ng) was reverse transcribed to cDNA using the Super Script VILO cDNA Synthesis kit (Invitrogen) according to manufacturer's instructions and diluted to the final concentration of 5 ng/μl. Quantification of gene expression for collagen type 1 alpha (*COL1A1)*, transforming growth factor beta 1 (*TGFbeta1*), interleukin 1 beta (*IL1beta*), matrix metallopeptidase 1 (*MMP1*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* (used as reference

gene) was performed in a LightCycler Instrument (Roche Diagnostics GmbH, Mannheim, Germany) with the use of the Quanti Tect SYBR Green PCR Master Mix (Qiagen). Primer details are reported in Table 1. The protocol included

- denaturation at 95°C for 15 min;
- 25 to 45 cycles of amplification (95°C, 15 s, appropriate annealing temperature for each target gene for 20 s and 72°C for 20 s);
- melting curve analysis to check for amplicon specificity.

Each sample was tested in duplicate. Data were collected using the LightCycler Software 4.1. Relative quantification was performed using the comparative threshold (Ct) method (ΔCt), where relative gene expression levels equal $2^{-\Delta\Delta Ct}$. Gene expression levels of the target genes were calculated by normalization to the reference gene GAPDH, using the cells untreated as calibrators.

2.6 *Supernatant Enzyme-Linked Immunosorbent Assay Measurements*

After 24, 48, and 72 h from laser irradiation, supernatants were collected for collagen type 1 (*COLL1*), *TGFbeta1*, and *prostaglandin* E_2 (PGE_2) determinations by Enzyme-linked Immunosorbent Assay (ELISA) kits following manufacturer's instructions (R&D Systems, Inc., Minneapolis, Minnesota, for PGE_2 and Boster Biological Technology Co, Fremont, California, for other proteins). For *TGFbeta1* assays, cell supernatants were chemically activated before testing by two serial steps: 1 N HCl for 10 min followed by 1.2 N NaOH with 0.5 M Hepes for 10 min.

The measured protein concentrations were normalized by DNA content.

2.7 *Statistical Analysis*

The Shapiro-Wilk Test was used to assess the normality of data. For scratched-wound area, cell viability, *COL1A1* and *TGFbeta1* gene expression, the differences between the laser irradiation protocols for each experimental time were analyzed using a one-way multivariate analysis of variance (MANOVA) and Tukey's honest significant difference *post hoc* test, while the differences between the three experimental times were evaluated with ANOVA with repeated measures and *post hoc* tests using the Bonferroni correction. For DNA content, release of *COLL1* and *TGFbeta1*, the differences between the laser irradiation protocols for each experimental time were analyzed using a Kruskall–Wallis H test and a Bonferroni-corrected Mann–Whitney U *post hoc* test, while the differences between the three experimental times were evaluated with a Friedman test followed by a Bonferroni-corrected Wilcoxon paired sign-rank test for each laser irradiation protocol. All comparisons were performed between laser-irradiated and nonirradiated groups; inter laser-irradiated group comparisons were performed if the former ones were significant. Statistical analyses were performed using the statistical software SPSS for Windows (version 18.0; 2009; SPSS Inc., Chicago, Illinois). The limit for statistical significance was set at $p < 0.05$.

3 Results

3.1 *In Vitro Micro Wound Healing*

Cells progressively participated in the healing process enabling a gradual wound closure (Fig. 1). The 5 and 10 J/cm^2 laser-treated groups were the first to reach complete wound closure after 72 h, followed by the 15 J/cm^2 laser-treated group after 96 h from irradiation. Nonirradiated controls still showed partial wound healing after 96 h (Fig. 1).

Table 1 Primer specifications.

Gene	Alias	Primer Fw (5' → 3')	Primer Rv (5' → 3')	T annealing	Product length	Target
GAPDH[a]	Glyceraldehyde-3-phosphate dehydrogenase	TGGTATCGTGGAAGGACTCA	GCAGGGATGATGTTCTGGA	55°C	123 bp	*NM_001289746.1 NM_001289745.1 NM_001256799.2 NM_002046.5*
COL1A1[b]	Collagen, type I, alpha	Hs_COL1A1_1_SG		55°C	118 bp	*NM_000088*
TGFbeta1[b]	Transforming growth factor, beta 1	Hs_TGFB1_1_SG		55°C	108 bp	*NM_000660*
IL1beta[b]	Interleukin 1, beta	Hs_IL1B_1_SG		55°C	117 bp	*NM_000576*
MMP1[c]	Matrix metallopeptidase 1	TGGACCTGGAGGAAATCTTG	CCGCAACACGATGTAAGTTG	56°C	125 bp	*NM_001145938.1 NM_002421.3 NM_133636.3 NM_001297756.1 NM_001297757.1*

[a]Designed with Primer Blast.[36]
[b]QuantiTect Primer Assay – Qiagen.
[c]Reference 37.

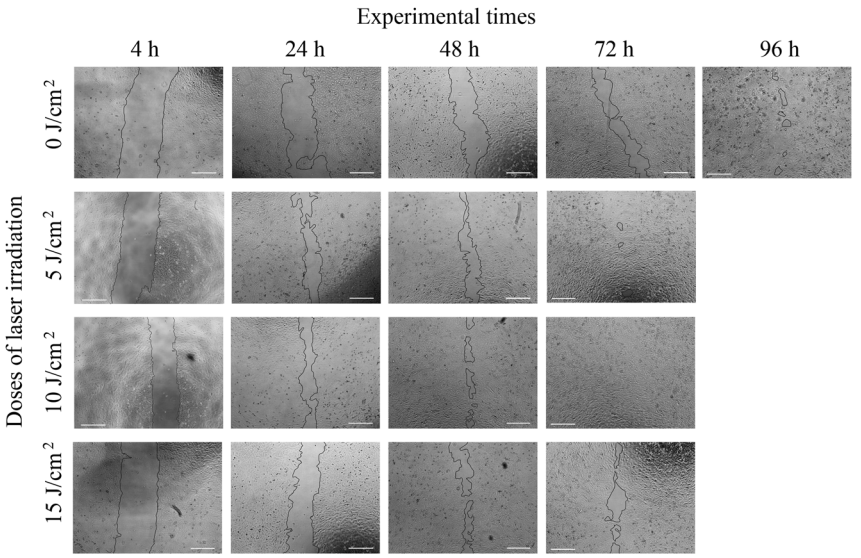

Fig. 1 Representative micrographs of the *in vitro* scratch wound of Saos-2 cells treated with different laser doses (5, 10, and 15 J/cm^2 or untreated (0 J/cm^2) at different times (4, 24, 48, 72, and 96 h). Black lines mark the residual wound area. Bar = 500 μm.

After 4 h, the 5 and 10 J/cm^2 laser-irradiated groups exhibited a significantly decreased wound area compared with the nonirradiated controls ($p < 0.0005$, Fig. 2). After 48 and 72 h, all the laser-irradiated groups exhibited a significantly decreased wound area compared with nonirradiated controls ($p < 0.0005$ for all, except for 15 J/cm^2 versus 0 J/cm^2 at 72 h with $p = 0.001$, Fig. 2) with a dose-dependent effect especially at 48 h (15 J/cm^2 versus 5 and 10 J/cm^2 with decreasing significance of $p < 0.0005$ and $p = 0.001$, respectively, Fig. 2). By analyzing data over the experimental times, the 5 J/cm^2 laser-irradiated group and nonirradiated controls significantly decreased the wound area between 4 and 72 h ($p = 0.008$, Fig. 2); in addition, control cell cultures significantly decreased the wound area between 4 and 24 h ($p = 0.008$, Fig. 2). The 10 J/cm^2 laser-irradiated group showed a significant decrease in wound area at almost all the experimental times (4 h versus 48 and 72 h, $p = 0.003$; 48 h versus 72 h, $p = 0.002$, Fig. 2).

Similar to other cell cultures, in the presence of MMC, the 10 J/cm^2 laser-irradiated group reached complete wound

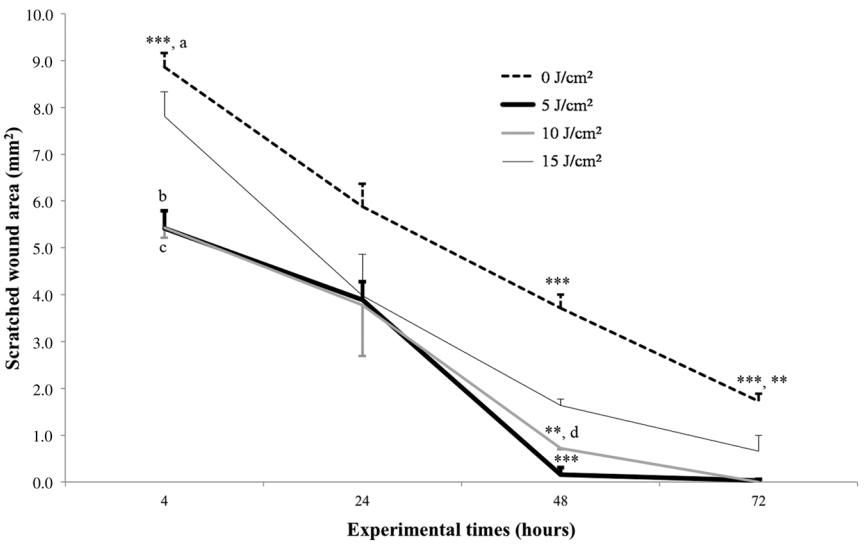

Fig. 2 Scratched wound area measured at different times (4, 24, 48, and 72 h) of Saos-2 cells treated with different laser doses (5, 10, and 15 J/cm^2) or untreated (0 J/cm^2). Data are means; bars are standard deviations. One-way multivariate analysis of variance (MANOVA) and Tukey's honest significant difference (HSD) *post hoc* tests: 4 h: ***, 0 J/cm^2 versus 5 and 10 J/cm^2, $p < 0.0005$; 48 h: ***, 0 J/cm^2 versus 5, 10, and 15 J/cm^2, $p < 0.0005$, ***, 5 J/cm^2 versus 15 J/cm^2, $p < 0.0005$, **, 10 J/cm^2 versus 15 J/cm^2, $p = 0.001$; 72 h: ***, 0 J/cm^2 versus 5 and 10 J/cm^2, $p < 0.0005$, **, 0 J/cm^2 versus 15 J/cm^2, $p = 0.001$. ANOVA with repeated measures and *post hoc* tests using the Bonferroni correction: 0 J/cm^2: a, 4 h versus 24 and 72 h, $p = 0.008$; 5 J/cm^2: b, 4 h versus 72 h, $p = 0.008$; 10 J/cm^2: c, 4 h versus 48 and 72 h, $p = 0.003$, d, 48 h versus 72 h, $p = 0.002$.

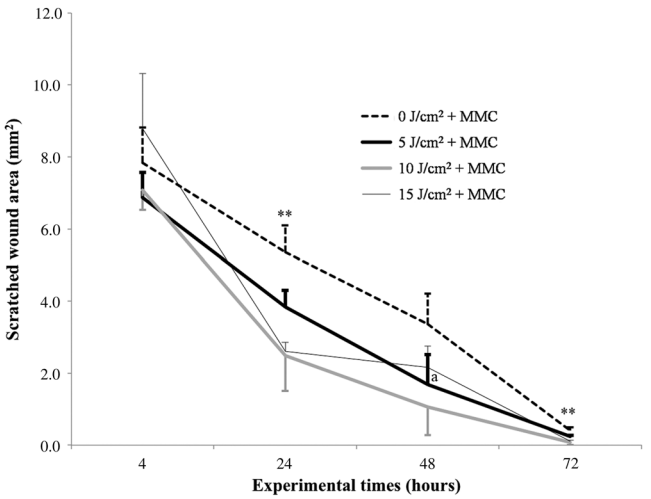

Fig. 3 Scratched wound area measured at different times (4, 24, 48, and 72 h) of Saos-2 cells treated with different laser doses (5, 10, and 15 J/cm^2) or untreated (0 J/cm^2) in the presence of Mitomycin C (MMC). Data are means; bars are standard deviations. One-way MANOVA and Tukey's HSD *post hoc* tests: 24 h: **, 0 J/cm^2 versus 10 and 15 J/cm^2, $p < 0.005$; 72 h: **, 0 J/cm^2 versus 10 and 15 J/cm^2, $p = 0.001$. ANOVA with repeated measures and *post hoc* tests using the Bonferroni correction: 5 J/cm^2: a, 48 h versus 4 h, $p = 0.001$.

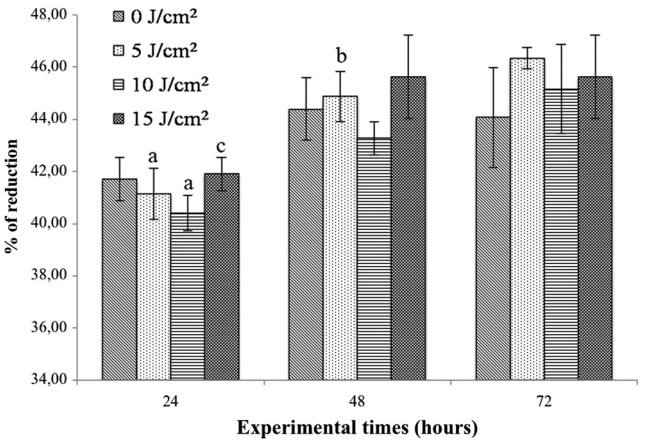

Fig. 4 Viability results of Saos-2 cells treated with different laser doses (5, 10, and 15 J/cm^2) or untreated (0 J/cm^2) after 24, 48, and 72 h from irradiation. Data are means; bars are standard deviations. ANOVA with repeated measures and *post hoc* tests using the Bonferroni correction: 5 and 10 J/cm^2: a, 24 h versus 48 and 72 h, $p < 0.0005$; 5 J/cm^2: b, 48 h versus 72 h, $p = 0.008$; 15 J/cm^2: c, 24 h versus 72 h, $p = 0.001$.

closure after 72 h and the 15 J/cm^2 laser-irradiated group healed after 96 h, while nonirradiated controls still showed partial healing after 96 h from scratch (Fig. 3). The 5 J/cm^2 laser-irradiated group, however, reached a complete wound closure after 96 h (with MMC) rather than after 72 h (without MMC). Statistically significant differences between the groups were only slightly less pronounced and more time-delayed in the presence of the cell proliferation inhibitor. The 10 and 15 J/cm^2 laser-irradiated groups had decreased wound area compared with nonirradiated controls after 24 h ($p = 0.003$ and $p = 0.004$, respectively) and 72 h ($p = 0.001$) (Fig. 3). Data analysis over the experimental times showed a statistically significant decrease in wound area only for the 5 J/cm^2 laser-treated group between 4 and 48 h ($p = 0.001$, Fig. 3).

3.2 *Viability Test and DNA Quantification*

There was no statistically significant difference in cell viability between laser-irradiated and nonirradiated groups for each experimental time (Fig. 4). Cell viability showed a statistically significant increase between 24 and 72 h for all laser-irradiated groups ($p<0.0005$ for 5 and 10 J/cm^2, $p=0.001$ for 15 J/cm^2), between 24 and 48 h for the 5 and 10 J/cm^2 laser-irradiated groups ($p < 0.0005$), and between 48 and 72 h for the 5 J/cm^2 laser-irradiated group ($p = 0.008$).

In agreement with the viability results, no statistically significant difference was found for DNA content between the different treatment protocols or experimental times.

3.3 *Quantification of mRNA Expression Levels by Quantitative Polymerase Chain Reaction*

After 24 h, *COL1A1* gene expression was increased in all laser-irradiated groups compared with nonirradiated controls ($p < 0.0005$, Fig. 5). Moreover, the 15 J/cm^2 laser-irradiated group showed an increased *COL1A1* gene expression compared with the 5 and 10 J/cm^2 laser-irradiated groups ($p = 0.001$, Fig. 5). The level of *COL1A1* gene expression showed a significant increment at 48 h in the 10 and 15 J/cm^2 laser-irradiated groups compared with the nonirradiated group and reached the highest values in the 10 J/cm^2 laser-irradiated group ($p < 0.0005$, Fig. 5). After 72 h, the 15 J/cm^2 laser-irradiated group showed an increased *COL1A1* gene expression compared with the nonirradiated group ($p < 0.005$, Fig. 5). Over the experimental times, nonirradiated controls showed an increased *COL1A1* gene expression at 72 h compared with 24 h ($p < 0.0005$, Fig. 5). The 10 J/cm^2 laser-irradiated group showed an increased *COL1A1* gene expression at 48 h compared with 24 h ($p < 0.005$, Fig. 5); the same trend was observed in the 15 J/cm^2 laser-irradiated group at 72 h compared with 48 h ($p < 0.005$, Fig. 5).

The gene expression of *TGFbeta1* showed no statistically significant differences after 24 h between the experimental conditions; after 48 h, nonirradiated controls showed an increased expression compared with the 5 J/cm^2 ($p = 0.008$) and the 15 J/cm^2 laser-irradiated groups ($p < 0.0005$) (Fig. 6). After 72 h, the 10 J/cm^2 laser-irradiated group exhibited a decreased *TGFbeta1* gene expression compared with the nonirradiated group ($p < 0.0005$) (Fig. 6). Over the experimental times, nonirradiated controls showed an increased *TGFbeta1* gene expression at 24 h compared with 48 h ($p < 0.0005$, Fig. 6); at 72 h, the *TGFbeta1* gene expression in the 10 J/cm^2 laser-irradiated group decreased compared with 24 h ($p = 0.001$) (Fig. 6).

Gene expression for *IL1beta* and *MMP1* was undetectable at each experimental time and condition.

3.4 *Supernatant ELISA Measurements*

No statistically significant differences were observed between laser-irradiated and nonirradiated groups for *COLL1* and *TGFbeta1* release. At a protein level, PGE_2 was undetectable at each experimental time and condition.

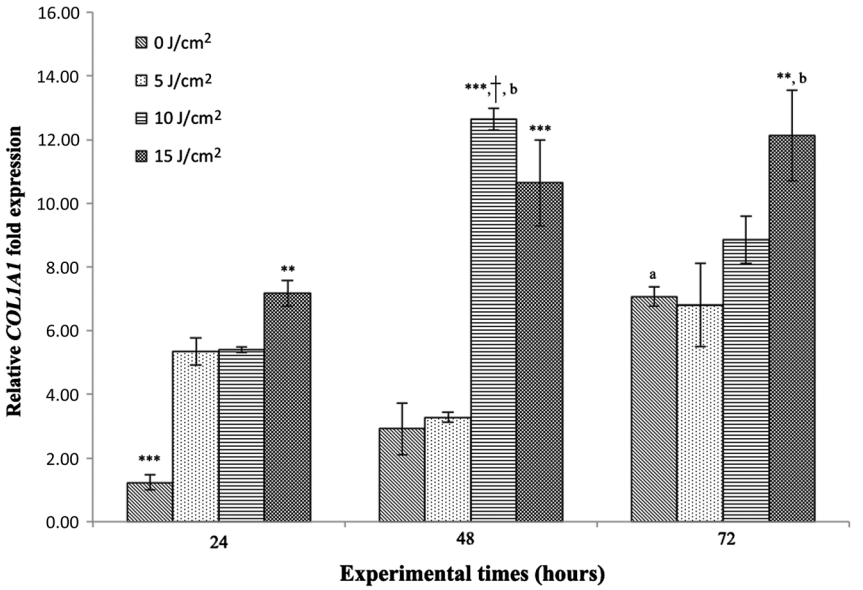

Fig. 5 Relative gene expression of *COL1A1* of Saos-2 cells treated with different laser doses (5, 10, and 15 J/cm^2) or untreated (0 J/cm^2) after 24, 48, and 72 h from irradiation. Data are means; bars are standard deviations. One-way MANOVA and Tukey's HSD *post hoc* tests: 24 h: ***, 0 J/cm^2 versus 5, 10, and 15 J/cm^2, $p < 0.0005$; **, 15 J/cm^2 versus 5 and 10 J/cm^2, $p = 0.001$. 48 h: ***, 10 and 15 J/cm^2 versus 0 J/cm^2, $p < 0.0005$; †, 10 J/cm^2 versus 15 J/cm^2, $p < 0.0005$. 72 h: **, 15 J/cm^2 versus 0 J/cm^2, $p < 0.005$. ANOVA with repeated measures and *post hoc* tests using the Bonferroni correction: 0 J/cm^2: a, 24 h versus 72 h, $p < 0.0005$; 10 J/cm^2: b, 24 h versus 48 h, $p < 0.005$; 15 J/cm^2: b, 48 h versus 72 h, $p < 0.005$.

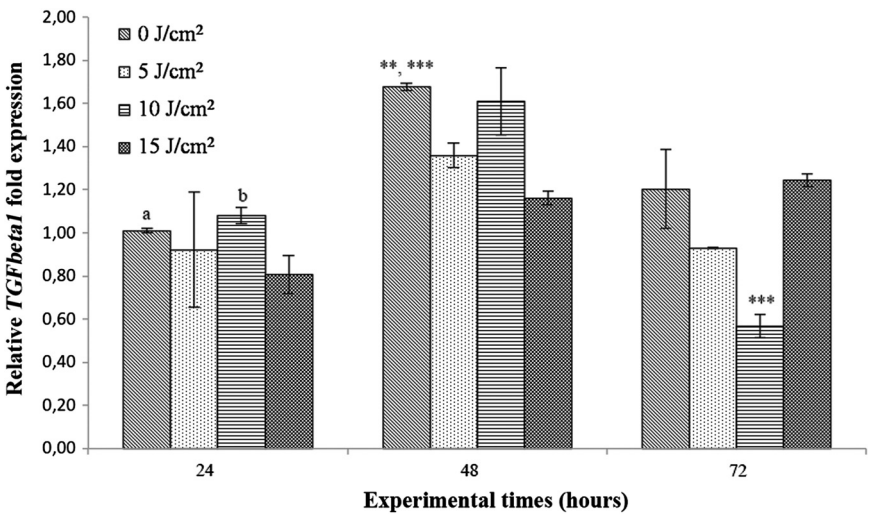

Fig. 6 Relative gene expression of *TGFbeta1* of Saos-2 cells treated with different laser doses (5, 10, and 15 J/cm^2) or untreated (0 J/cm^2) after 24, 48, and 72 h from irradiation. Data are means; bars are standard deviations. One-way MANOVA and Tukey's HSD *post hoc* tests: 48 h: **, 0 J/cm^2 versus 5 J/cm^2, $p = 0.008$; ***, 0 J/cm^2 versus 15 J/cm^2, $p < 0.0005$. 72 h: ***, 10 J/cm^2 versus 0 J/cm^2, $p < 0.0005$. ANOVA with repeated measures and *post hoc* tests using the Bonferroni correction: 0 J/cm^2: a, 24 h versus 48 h, $p < 0.0005$; 10 J/cm^2: b, 24 h versus 72 h, $p = 0.001$.

4 Discussion and Conclusions

To our knowledge, this is the first study that examines the effects of irradiation with a 915-nm GaAlAs diode laser on osteoblast migration and proliferation using an *in vitro* scratch-wound healing assay. This method has already been described as a convenient and inexpensive way to measure the cell healing capacity *in vitro*.[38] Moreover, culture treatment with MMC allowed us to investigate the relative contribution of cell migration and proliferation to the process of scratch-wound closure. A Saos-2 human osteosarcoma-derived cell line was employed due to its resemblance to human mature osteoblast phenotype and its key role in bone healing, repair and remodeling.[39,40] Unlike other human osteosarcoma-derived cell lines, such as U2OS cells used by Spitler and Berns,[27] the cell line chosen

for this study is able to differentiate and form calcified matrix and more closely resembles the osteoblast profile as far as the expression of bone remodeling proteins is concerned.[34] A diode laser was employed in this study because it is one of the most popular in clinical practice and, at a wavelength of 915 nm, it is known to have a high penetration depth that is desirable for clinical applications on bone.[2,41]

Cells irradiated with a single laser application administered at doses of 5, 10, and 15 J/cm^2 showed an increased healing ability compared with nonirradiated controls. Nonirradiated controls still showed partial healing after 96 h, while the 5 and 10 J/cm^2 laser-irradiated groups were the first to reach complete wound closure after 72 h. The 15 J/cm^2 laser-irradiated group reached complete closure after 96 h and showed a tendency toward increased wound area compared with that of other laser-treated groups at each experimental time, suggesting a decreased healing ability for this dose of irradiation. When MMC was added to the culture medium, thus abolishing the contribution of cell proliferation, only the 5 J/cm^2 laser-irradiated group reached a complete wound closure in a time-delayed manner (after 96 h rather than after 72 h). Accordingly, with MMC, the 5 J/cm^2 laser-irradiated group failed to show a statistically significant decreased wound area, while the 10 and 15 J/cm^2 laser-irradiated groups maintained a decreased wound area compared with nonirradiated controls. These results suggest that laser irradiation with a wavelength of 915 nm promotes the closure of the scratched wound area mainly through stimulation of Saos-2 cell migration, in accordance with a previous report on human osteosarcoma cells using laser wavelengths of 652 and 806 nm.[27] Previous studies have demonstrated the ability of PBM to stimulate proliferation and migration of many other cell phenotypes, but direct comparison with the current data is inappropriate due to different *in vitro* conditions.[30,32,33,42]

A significant increase in cell viability over the experimental times was detected for laser-treated groups and not for nonirradiated cells, but no statistically significant differences were found between laser-irradiated and nonirradiated groups. A previous report on healthy Saos-2 cells using the same laser equipment and parameters adopted in the present study concluded that, at 72 h from irradiation, the 10 J/cm^2 treated group showed a significantly higher viability compared with the nonirradiated controls.[43] Probably, these contradictory results can be ascribed to the different *in vitro* models adopted.

In accordance with the viability assays, laser irradiation had no statistically significant effect on DNA content. On the other hand, laser irradiation showed an influence on cellular anabolic properties through modulation of *COL1A1* and *TGFbeta1* gene expression. *COL1A1* is a major protein in the bone extracellular matrix and it is intimately related to the achievement of bone tissue healing. Nonirradiated cells exhibited a constantly increasing *COL1A1* gene expression over time; laser irradiation significantly increased its gene expression, reaching statistically significant differences for the 5, 10, and 15 J/cm^2 groups at 24 h and for the 10 and 15 J/cm^2 groups at 48 h, as well as for the 15 J/cm^2 group at 72 h compared with nonirradiated controls. These data suggested that a higher dose induced a longer-lasting effect on *COL1A1* gene expression in the range between 5 and 15 J/cm^2. This trend was confirmed by the protein analyses, although no statistically significant differences were detected. Despite the different laser equipment and parameters used, our results are consistent with those of other studies, which found an increased expression for this gene after PBM on mouse fibroblasts,[44] human gingival fibroblasts,[45] rat bone tissue,[46] human keratinocytes,[42] and porcine Achilles tendon fibroblasts.[47]

A slightly more delayed effect of laser irradiation was found on the gene expression of *TGFbeta1*, a potent cytokine that acts as a leading factor in the process of bone healing. Compared with nonirradiated cells, the 5 and 15 J/cm^2 laser-irradiated groups showed a decreased *TGFbeta1* gene expression at 48 h, whereas only the 10 J/cm^2 laser-irradiated group retained comparable gene expression and then significantly decreased. Previous studies showed an enhanced *TGFbeta1* production after low-level laser irradiation on osteoblast-like cells, but without performing the *in vitro* scratch-wound healing assay.[48] Other reports supported the idea that its secretion after laser and LED irradiation decreases both *in vivo* on rats and *in vitro* on human umbilical vein endothelial cells.[49,50] Moreover, *TGFbeta1* levels have been shown to follow a phasic expression pattern over time postwounding in a clinical oral tooth extraction healing study.[23] However, these biological effects seemed to be dependent on the individual cell phenotype as well as on the irradiation parameters, mainly wavelength, power density and irradiation time.[2,49,50] Therefore, direct comparison with the current study is inappropriate because of different laser equipment, treatment protocol, *in vitro* model, and conditions used. In our experiment, at early experimental times, only the 10 J/cm^2 laser-irradiated group maintained a *TGFbeta1* gene expression similar to untreated controls, while the 5 and 15 J/cm^2 laser-irradiated groups showed decreased gene expression, suggesting a biphasic dose response of osteoblasts-like cells treated with laser.[33]

The present study investigated the effects of a single session of laser irradiation on wound healing; the output power, the pulsing of the radiation, and the treated area were kept constant while varying the dose as the main study variable because it has been recognized as the most important laser parameter responsible for the biologic response.[3] Further investigations with multiple sessions of laser irradiation should strengthen cell responses owing to a cumulative laser effect.[51] Our results indicate that the *in vitro* scratch-wound healing assay induced a mechanical injury without an altered inflammatory status because of the lack of gene expression of catabolic and inflammatory proteins, such as *IL1beta* and *MMP1* and because of the lack of PGE_2 production. For further investigations, it might be useful to employ primary human pathological cells and to add inflammatory cytokines to the culture medium in order to provide an *in vitro* environment that is closer to that of actual wound healing. Rigorous *in vitro* studies on the cellular and photobiological mechanisms of laser irradiation might be helpful to bridge the gap between *in vitro* research and biomedical applications.

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Biographies for the authors are not available.